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High Resolution Light Microscopy and Immunocytochemistry Using Glycol Methacrylate-Embedded Sections and Immunogold-Silver Staining.

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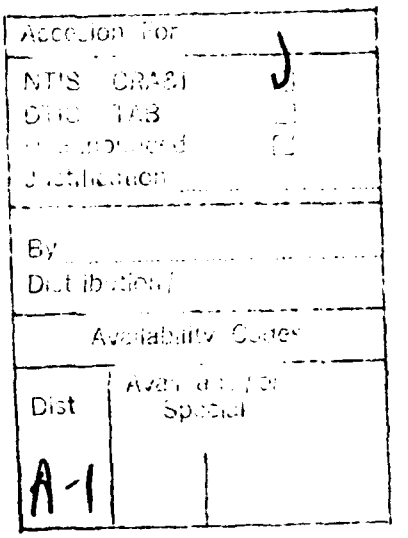

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Abstract

The immunogold-silver technique was modified so that 1-2 micron-thick glycol methacrylate sections could routinely be used for high resolution immunocytochemistry. A rabbit anti-human hemoglobin antibody was used to demonstrate the distribution of exogenous hemoglobin in mouse tissues. Inclusion of 0.1% glutaraldehyde in the primary formalin fixation improved tissue appearance. Glutaraldehyde treatment after colloidal gold antibody application increased the staining intensity, and gold chloride intensification of the silver-enhanced colloidal gold further improved immunosensitivity. Thus a more accurate analysis of hemoglobin distribution in tissue was obtained. The benefit of these enhancements was also demonstrated for localizing myelin basic protein in brain and for determining the proliferative status of tissue by localization of bromodeoxyuridine incorporation into nuclei.

The efficiency of manual capillary-action staining performed during all steps in the immunogold-silver staining protocol was also demonstrated. This greatly simplified the procedure, reduced technician time, and produced better staining consistency. The immunogold-silver staining technique is simple, sensitive, economical, and relatively nontoxic.

Key Words: capillary-action staining, glutaraldehyde postfixation, glycol methacrylate sections, gold chloride intensification, immunogold-silver staining

High Resolution Light Microscopy and Immunocytochemistry Using Glycol Methacrylate-Embedded Sections and Immunogold-Silver Staining. [1]

Although plastic-embedded tissue offers high resolution and good histological detail of 1-2 micron thick sections, it presents a special problem with immunochemical procedures because the plastic limits the access of the tissue to the reagents. Immunoperoxidase and enzyme labeling techniques for glycol methacrylate (GMA), Araldite, and Spurr's epoxy resin have been described (1-3). However, these procedures are either complicated and time consuming or yield only light immunostaining. Diaminobenzidine (DAB), the most common chromogen used in plastic and paraffin immuno-techniques, is a suspected carcinogen, and requires special handling. Vinyl cyclohexene dioxide in Spurr's epoxy resin is a known potent carcinogen, and other epoxy resins used in electron microscopy (Araldite and Epon 812) should also be treated as suspected carcinogens (4). GMA on the other hand, has not been reported to be carcinogenic.

Although the immunogold-silver staining (IGSS) procedure is sensitive and has been used successfully in frozen, paraffin, and plastic sections (5-9), it is not commonly used with GMA. It has been reported to enhance sensitivity up to 200-fold compared with standard immunoperoxidase staining methods (5). The method is also easily reproduced and the reagents used with it have low toxicity.

This report describes a simple IGSS staining technique in which

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[1] The opinions and assertions contained herein are the private views of the authors and are not to be construed as official nor do they reflect the views of the Department of the Army or the Department of Defense (AR 360-5)

intense and discrete immunostaining is achieved in standard GMA sections. Histologic detail is well maintained for high resolution light microscopy. A test system in which human hemoglobin was administered to mice was developed to unambiguously detect the presence of a foreign antigen in well defined tissues. Antibodies against human myelin basic protein in mice and bromodeoxyuridine (BrDU) incorporation into rabbit intestine to assess cell proliferation were also used in testing the IGSS technique. The capillary action staining system developed by Brigati et al (10) was used to perform all stages of the procedure with increased speed of immunostaining and decreased background staining.

Materials and Methods

Tissue and Slide Preparation

Three to six month old (25-30 gm) male Balb C mice were used. Specimens were prepared by tail vein injection of 100 microliters of high pressure liquid chromatography-purified human hemoglobin (HbAo; 25 gm/dl) (11). Thirty minutes after injection, mice were sacrificed and tissues fixed in 2% paraformaldehyde-0.1% glutaraldehyde for 4-6 hr at room temperature or overnight at 4°C (12). In one experiment, a single 6 kg rabbit was injected intraperitoneally with 20 ml of BrDU/fluorodeoxyuridine (Amersham, Arlington Heights, IL), sacrificed after 2 hr, and the tissue fixed as described above. Tissues for GMA embedding were dehydrated in a graded series of ethanol and then infiltrated and embedded in HistoResin glycol methacrylate (Leica, Deerfield, IL) at 4°C according to manufacturer's instructions. (JB-4 glycol methacrylate can be substituted.) Sections were cut at 1-2 μm with glass knives on a Reichert/Jung 2040 Autocut rotary microtome (Leica). Sections were floated on a waterbath of distilled water,

placed on Probe-On slides (Fisher Scientific; Pittsburg, PA), and dried on a hotplate at 60-70^{o}C for 15-30 min. Tissues for paraffin embedment were processed with a Fisher MVP Tissue Processor.

Immunocytochemistry

Antiserum

Anti-human hemoglobin antibody was prepared by injecting 2 rabbits with purified HbAo in complete Freund's adjuvant and injecting them again 21 days later with the same in incomplete Freund's adjuvant. Ten days later the rabbits were bled and serum prepared (12). The polyclonal antiserum against hemoglobin was adsorbed with mouse spleen powder (Sigma) for 1 hr at 37^{o}C and overnight at 4^{o}C. Supernatant was recovered and titrated to the desired dilution. Polyclonal anti-myelin basic protein (MBP; Chemicon, Temecula, CA) was used for immunolocalization of this antigen in mouse brain. Anti-BrdU reagents were purchased from Amersham and used according to the manufacturer's directions.

Immunohistochemical Reagents

Blocking solution was prepared as follows: Casein buffer (CAS) was prepared by stirring 5 gm of purified casein powder (Sigma; St. Louis, MO) and 0.5 gm of thimerosal (Sigma) in 1000 ml of Dulbecco's phosphate buffered saline (PBS) (Sigma) (12). The suspension was stirred overnight and the pH was adjusted to 7.4. The 0.5% CAS was slightly turbid and was stored at 4^{o}C. Anti-HbAo antibody was diluted 1:500 in CAS. As an alternative, 1% Bovine serum albumin or fish gelatin in PBS containing 0.25% Tween could also used with similar results.

Immunocytochemistry

Immunoperoxidase staining of sections was performed using the ABC Elite kit (Vector Laboratories; Foster City, CA). Development of the

peroxidase reaction was achieved by incubating sections in DAB substrate solution (25 mg/50 ml) containing 5 drops of 3% hydrogen peroxide and 3% nickel chloride-cobalt chloride for 5-10 min.

Immunogold staining was accomplished with goat anti-rabbit-coated 5 nm colloidal gold purchased from BioCell (Ted Pella, Redding, CA) diluted 1:50 in PBS containing 0.25% Tween and 0.1% fish gelatin. IntenSE Silver Enhancer (Janssen) was purchased from Amersham. Gold chloride, 1% aqueous, was purchased from VWR Scientific (San Francisco, CA).

Manual Capillary Action System with the MicroProbe staining system (Fisher Scientific)

Tissue sections on MicroProbe slides--which had a 75 μm-thick coating of paint covering the label and the lower corners of each slide--were placed face to face in their slide holder. The thickness of the painted portions created a capillary gap of 150 μm, which was filled by dipping the ends of the apposed slides into a reagent bucket and/or rubber isolator (Isolon), thus exposing tissues to the reagent. Slides were drained by placing them in contact with an absorbent pad. The gap was filled with 150-200 μl of reagent, and up to 20 slides could be stained simultaneously with each MicroProbe slide holder. To facilitate the movement of reagents in and out of the capillary gap, 0.25% Tween 20 was added to reagents.

IGSS Method

All steps are performed with the MicroProbe staining system. Tween 20 (0.25%) is added to all reagents and antibodies, except those used for the silver-enhancement step and organic solvents.

1. Incubate with 0.5% casein buffer for 5 min at 42°C to block nonspecific background staining. Blot. (Substitute 5% normal goat serum for 15-20 minutes.) (Optional step).
2. Incubate with primary antibody for 30 min to 1 hr at 42°C, or overnight at 4°C.
3. Wash 4 times in PBS.
4. Incubate with 1:50 anti-rabbit/mouse (dependent on primary antibody) conjugated-gold for 30-45 min at 42°C.
5. Wash 4 times in PBS.
6. Post-fix in 2% glutaraldehyde in PBS for 10 min at 42°C.
7. Wash 1 time in PBS.
8. Wash 4 times in distilled water-Tween 20.
9. Enhance with silver according to the suppliers instructions for 5-10 min

   Steps 9 can be repeated at 2-4 minute intervals after the completion of step 12.
10. Wash 2 times in distilled water.
11. Apply gold chloride (0.01%) intensifier for 5-10 sec.
12. Wash 1 time in distilled water.
13. Counterstain with 3X Gill's hematoxylin for 1 min. Blot.
14. Wash 3-4 times in distilled water.
15. Apply saturated lithium carbonate for 15-30 sec. Blot.
16. Dehydrate, clear in xylene, and coverslip with Accu.Mount 60™ mounting medium.

Results

In 1-2 micron-thick GMA sections, histological preservation was excellent. Exogenous hemoglobin visualized in tissue by DAB staining, however, was only light and diffuse (Figure 1). Sections from the same block stained by the IGSS method showed improved immunostaining (Figure 2). Further improvements in the IGSS method are shown in Figure 3. Postfixing after application of the colloidal gold obtained a more intense signal. A further enhancement was obtained by application of 0.01% gold solution which further darkened the silver-enhanced colloidal gold (Figure 3B). The improved IGSS method clearly delineated the basement membranes of arterioles, the proximal tubules and the glomerular tuft. The stain was diffuse across the microvillar border and densely concentrated in the numerous discrete vacuoles in the lumenal cytoplasm. This procedure revealed that by 30 minutes, exogenously administered hemoglobin was localized in kidney tissue. Lack of significant background staining with this method is shown in Figure 3C in which primary antibody was omitted. Morphologic detail in the proximal tubules was particularly clear (Figure 4A). In contrast, morphological detail was compromised in thicker paraffin sections (Figure 4B). Peroxidase stained paraffin sections showed a diffuse and modest reaction of hemoglobin at the region of the proximal tubular brush border and in the lumens of the distal tubular cells.

The use of the MicroProbe capillary action staining system considerably simplified the immunostaining procedure. Extraneous silver precipitation which often occurs at the edges of the sections by the drop method (Figure 5A), was eliminated by the capillary gap method (Figure 5B). Because less reagent was used with this method, the cost was reduced.

Technician time was also reduced by 70-80%, and a large number of slides (20-40) were easily stained at one time.

The applicability of this system to other antigen-antibody systems is also illustrated in this report. Figure 6 illustrates the localization of myelin basic protein in mouse brain and Figure 7 illustrates the nuclear localization of BrDU in rabbit intestine. In both systems, high resolution and clear delineation of antigen was apparent.

Discussion

Purified human hemoglobin (HbAo) has been proposed to serve as an oxygen-carrying blood substitute and we were particularly interested in the histologic localization of hemoglobin in the kidney. Attempts to visualize the HbAo with a polyclonal anti-human hemoglobin antisera using an immunoperoxidase detection system yielded ambiguous results.

Using aldehyde fixed tissues, GMA-embedment and 1-2 micron sections in conjunction with the IGSS method achieved the high resolution and excellent morphological preservation needed to precisely ascertain clear and accurate localization of HbAo in tissue.

Initially, LR White methacrylate (Polysciences, Warrington, PA) was considered as a media for embedding tissues. LR White has been primarily used in immunoelectron-microscopy techniques, but has also been utilized with an IGSS method in light microscopy prior to electron microscopy (7). However, it is well documented that LR White plastic sections have difficulty in adhering to glass slides, and a loss of sections is a problem when they are exposed to detergents such as Tween 20 (13, 14). GMA sections, on the other hand, adhered to slides when dried on a hotplate at 60-70^{0}C for 15-30 minutes.

We also compared the two-step indirect gold-labeling technique (used in our protocol) to a streptavidin-gold-biotin bridge technique. In the two-step indirect method, 1:50 anti-rabbit conjugated gold IgG (BioCell) was used to link up with the primary antibody, and in the streptavidin-biotin bridge, 1:50 biotinylated anti-rabbit IgG (Elite Kit, Vector Laboratories; Foster City, CA), was followed by 1:50 streptavidin-gold (BioCell) to link up with the biotinylated secondary IgG. Immunostaining improved with the two-step method, which

also simplified the procedure, reduced reagent cost, and reduced technician time.

Post-fixation (2% glutaraldehyde) immediately after the application of the gold-antibody complex (13), intensified immunogold staining. (Figure 3A and B). Post-fixation with Formalin versus glutaraldehyde was compared at various concentrations (1%-2%) and times (1, 5, 10, 15, and 30 minutes); our results clearly showed that glutaraldehyde fixation was superior to formalin (data not shown). The intensity of immunostaining also varied with the length of time sections were exposed to glutaraldehyde. For our system, 10 minutes at 42^{0}C was optimum for immunostaining.

The silver enhancement of the colloidal gold is also time-dependent. The 5 nm conjugated-gold particles are amplified by the precipitation of silver; however, if sections are over-exposed, silver precipitates may cause high background over the entire section. This spontaneous reaction (self nucleation) can happen very quickly (1-2 minutes) after optimum silver enhancement and therefore one must determine a safe margin of optimum enhancement versus high background staining (15).

A further enhancement of the gold-silver reaction was achieved by incubating sections in 0.01% gold chloride (0.1% for paraffin sections) after silver enhancement. The metallic-silver reaction of the silver enhancement step may appear yellowish brown to black depending on the amount of silver present and the particle size. These yellowish brown or black deposits may be transformed or "toned" to a dark black deposit with the use of gold chloride (15). The toned sections show better contrast and clarity than untoned sections (Figures 3B and 4A & B).

In conclusion, the described technique is useful for both research and diagnostic applications. The combination of high resolution GMA sections

with immunogold-silver staining produced exquisite cellular detail with good immunostaining. Immunolocalization was much more precise compared to conventional paraffin-immunoperoxidase methods. Endogenous enzyme activity poses no problem with this system. This method is applicable to other antibodies and protocols.

Immunostaining with a capillary action staining system produced an optimum combination of high quality immunostaining with reproducibility and technical efficiency. The capillary action staining system is relatively inexpensive and has replaced the conventional "drop method" formerly used in our laboratory.

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The experimental studies of the author described in this report were reviewed and approved by the Institutional Review Committee/Animal Care and Use Committee at Letterman Army Institute of Research. The manuscript was peer reviewed for compliance prior to submission for publication. In conducting the research described here, the author adhered to the "Guide for the Care and Use of Laboratory Animals," DHEW Publication (NIH) 85-23.

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Legend

Figure 1. Mouse kidney tubules stained with anti-hemoglobin antisera. Note the light brownish staining reaction. Tissue was embedded in GMA, 1-2 micron sections cut, followed by immunoperoxidase peroxidase-DAB detection system. Section was counterstained with hematoxylin. Original magnification x 400.

Figure 2. Mouse kidney stained with anti-hemoglobin antisera. Tissue was embedded in GMA, 1-2 micron sections cut, followed by the IGSS protocol. Section was counterstained with hematoxylin. Original magnification x 400.

Figure 3 Mouse kidney stained with anti-hemoglobin antisera. Tissue was embedded in GMA, 1-2 micron sections cut, followed by the IGSS protocol but some steps were omitted to illustrate benefit of enhancement procedures. All sections were counterstained with hematoxylin. Original magnification x 400.

(A) IGSS protocol without treatment with 2% glutaraldehyde or gold chloride enhancement. Light brownish-grey reaction is apparent. Compare with Figure 2 which used glutaraldehye and gold chloride enhancement.

(B) IGSS protocol without gold chloride enhancement. Brownish to grey-black reaction is apparent. Staining intensity is significantly improved with gold chloride enhancement. (Compare to Figure 2).

(C) Control. PBS was substituted for the primary antibody. No staining was apparent.

Figure 4. Mouse kidney tubules stained for HbAo. Sections were counterstained with hematoxylin. Original magnification x 1000 (oil immersion used).

(A) GMA section of kidney stained for HbAo with the IGSS method. Notice the discreet staining reaction of HbAo. Morphology and resolution is superior when compared to **(B).**

(B) Paraffin section of kidney stained for HbAo with an immunoperoxidase staining method (see Materials and Methods). Diffuse staining reaction of HbAo is apparent.

Figure 5 GMA embedded sections of mouse kidney stained for HbAo with IGSS. Original magnification X 100.

(A). IGSS was performed by placing drops of antibody and silver enhancement directly onto the sections. Note the heavy silver precipitation at the edge of the section.

(B) Silver precipitation artifact was eliminated by using the capillary action staining system.

Figures 6. GMA embedded mouse brain stained for MBP with IGSS. Section was counterstained with hematoxylin. Original magnification x 200.

Figure. 7. GMA embedded rabbit intestine stained for BrdU with IGSS. Section was counterstained with hematoxylin. Original magnification x 400. Note black reaction product in nuclei.